OUTER MEMBRANE PROTEINS OF *ESCHRICHIA COLI*: BIOSYNTHESIS AND ASSEMBLY

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Received 13 November 1973
(Revised version received 3 December 1973)

1. Introduction

Recently we have shown that mRNAs for some major membrane proteins of *Escherichia coli* are more stable than those of cytoplasmic proteins [1]. The mRNA for a structural lipoprotein of molecular weight 7000 was extraordinarily stable [1, 2].

In the present paper, we investigated the location of those membrane proteins produced by stable mRNAs in the *E. coli* envelope. We found that all membrane proteins produced by stable mRNAs exist in the outer membrane of *E. coli*. On the contrary, it was found that mRNAs for the inner (cytoplasmic) membrane proteins are as unstable as mRNAs for the cytoplasmic proteins. We have also attempted to investigate the assembly me chanism of the outer membrane proteins by pulse label experiments.

2. Methods

2.1. *Strains*

E. coli MX74T2 (thy^-, thi^-) was used.

2.2. *Labeling of the E. coli membrane proteins in the presence of rifampicin*

E. coli MX74T2 was grown at 37°C in 75 ml M9 medium supplemented with 4 mg/ml glucose, 4 μg/ml thymidine, 10 μg/ml L- arginine and 2 μg/ml thiamine. At about 1×10^8 cells/ml, 2.5 μCi of [^{14}C] arginine was added and the culture was incubated for 1.5 hr to about 3×10^8 cells/ml. Then rifampicin (Calbiochem) was added to the final concentration of 200 μg/ml. Five minutes after the addition of rifampicin, 50 μCi [^{3}H] arginine was added and the mixture was incubated for another 1.5 min. After the incubation, the mixture was immediately cooled in a dry ice–acetone mixture. The cells were collected by centrifugation.

2.3. *Pulse-label experiment*

A 100 ml culture of *E. coli* MX74T2 was grown in M9 medium as above. At about 1×10^8 cells/ml, 2.5 μCi [^{14}C] arginine was added and the culture was further incubated for 1.5 hr to about 3.2×10^8 cells/ml. Then 50 μCi [^{3}H] arginine was added to the culture and the mixture was incubated for 10 sec. Immediately after the pulse-label, the culture was cooled in a dry ice–acetone mixture.

2.4. *Miscellaneous procedures*

The outer and inner membranes were separated according to the method of Osborn et al. [3]; the inner membrane fraction in the present study corresponds to L1 and L2 fractions of their method. The ratio of the protein recovery in the outer membrane fraction to that in the inner membrane fraction was between 2.5 and 3.5 on the basis of radioactivity incorporated.

SDS-gel electrophoresis was carried out with 7.5% acrylamide gels as described previously [4, 5]. All gel-electrophoreses were run with internal molecular weight standards [6].

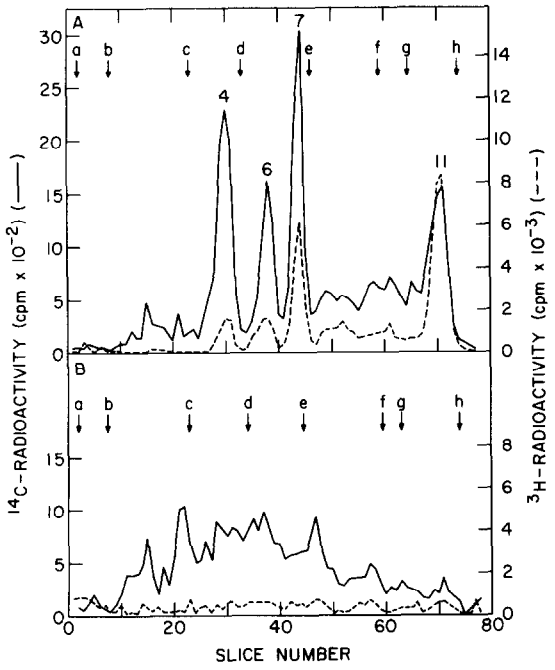

Fig. 1. Effects of rifampicin on the biosynthesis of outer and inner membrane proteins. *E. coli* MX74T2 (thy$^-$) was first labeled with [^{14}C] arginine for 1.5 hr at 37°C. Then rifampicin (200 μg/ml) was added to the culture. Five minutes after the addition of rifampicin, 50 μCi [^{3}H] arginine was added and the mixture was incubated for another 1.5 min as described in Methods. Membrane fractions were separated and their protein components are analyzed by SDS-gel electrophoresis as described previously [4, 5]. A) the outer membrane. B) the inner membrane. (———) [^{14}C] arginine; (·····) [^{3}H] arginine (+ rifampicin). Arrows with letters indicate positions of internal molecular weight standards [6]: a, trimer; b, dimer; c, monomer of l-dimethylaminonaphthalene-5-sulfonyl (DANS) bovine serum albumin; d, trimer; e, dimer; f, monomer of DANS–hen egg white lysozyme; g, cytochrome *c*; h, DANS–insulin. The number on each peak corresponds to those in the previous paper [6].

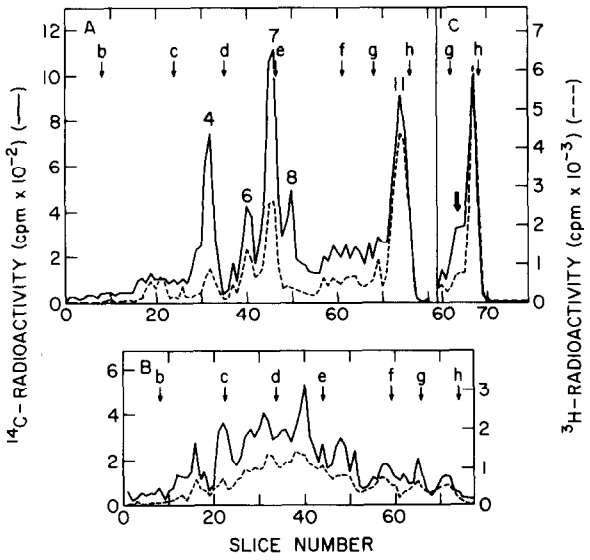

Fig. 2. Gel electrophoresis of the outer and the inner membrane pulse-labeled for 10 sec with [^{3}H] arginine. *E. coli* MX74T2 was labeled with 2.5 μCi [^{14}C] arginine for 1.5 hr. Then 50 μCi [^{3}H] arginine was added to the culture and the mixture was incubated for 10 sec as described in Methods. Immediately after the pulse-label, the culture was cooled in a dry ice–acetone mixture. The outer and inner membrane fraction were separated and then subjected to SDS-gel electrophoresis as in fig. 1. A) The outer membrane; B) the inner membrane; C) the outer membrane treated with T4 phage lysozyme before SDS-gel electrophoresis as previously described [7]. The gel pattern shows only the peak 11 region (between internal standard g and h). A large arrow indicates the appearance of the bound form of the lipoprotein (———) [^{14}C] arginine; (·····) [^{3}H] arginine (pulse-label). Assignments of internal standards of molecular weights (arrows with small letters) are the same as in fig. 1.

3. Results and discussion

It has been shown that substantial amounts of *E. coli* membrane proteins are still biosynthesized even after 5 min incubation of the cells in 200 μg/ml rifampicin [2]. Fig. 1. shows the location of these rifampicin-resistant membrane proteins in the *E. coli* envelope. Even after 5 min incubation in 200 μg/ml rifampicin, some of the outer membrane proteins are still biosynthesized (fig. 1A), whereas the biosynthesis of all the inner membrane proteins is severely inhibited (fig. 1B). It is evident that all the membrane proteins previously shown to be produced by stable mRNAs (peak 4, 6, 7 and 11) [2] exist in the outer membrane. It should be noticed that peak 11 protein which has been well characterized as a free form of a structural lipoprotein of molecular weight, 7000 [7, 8] exists almost exclusively in the outer membrane. In fig. 1A, the production of peak 11 protein is still 70% of the normal biosynthesis.

It appears that the biosynthesis of the inner membrane proteins is as sensitive to rifampicin as that of

the cytoplasmic proteins [2]. The present results suggest that the outer membrane proteins are biosynthesized somewhat differently from the other cellular proteins. Thus, we carried out studies on the assembly mechanism of the outer membrane proteins. A culture of *E. coli* MX74T2 was first labeled with [^{14}C] arginine for one generation. The culture was pulse-labeled with [^{3}H] arginine for 10 sec, and then immediately cooled in dry ice–acetone. The outer and inner membrane fractions were separated and their proteins are analyzed as described above. As shown in fig. 2, pulse-labeled ^{3}H-radioactivity appears almost parallel with ^{14}C in almost all peaks in both the outer and inner membrane fractions. This indicates that newly synthesized outer membrane proteins (^{3}H-labeled) are not accumulated in the inner membrane before they are incorporated into the outer membrane. Namely no large ^{3}H-labeled peaks which coincide with peak 4, 6, 7 and 11 of the outer membrane cannot be detected in the inner membrane fraction, when peaks in fig. 2B are compared with those in fig. 2A from their relative position to the internal standards. Even if 3×10^{-3} M dinitrophenol was added immediately after the 10 sec pulse-label, the same results were obtained (data not shown). At this concentration of dinitrophenol, amino acid incorporation was immediately stopped. These results suggest that partition coefficients of the outer membrane proteins in the outer membrane may be much higher than that in the inner membrane, so that the outer membrane proteins may not be detected in the inner membrane by the present method. Alternatively, the outer membrane proteins may be directly incorporated into the outer membrane from the site of their biosynthesis by an unknown mechanism.

The ratios of ^{3}H- to ^{14}C-radioactivities fall between 2.5 and 3.5 in almost all peaks in fig. 2. However, peaks 4 and 8 in the outer membrane show much lower ratios of ^{3}H- to ^{14}C-radioactivities (1.5 and 0.8 for peaks 4 and 8, respectively), whereas peak 11 shows a much higher ratio (=6). The reason for the low-ratios in cases of peaks 4 and 8 is not well understood. However, it may be because the proteins at peaks 4 and 8 are derived from a large pool of their own stable precursors, which may exist in the cytoplasmic fraction. In the case of the lipoprotein at peak 11 it has been shown that when the cells are pulse-labeled, only the free form is produced and eventually one third of the free form is converted into the bound form which is covalently linked to the peptidoglycan [7]. Therefore, the high ^{3}H to ^{14}C ratio of peak 11 can be explained by this unsteady state situation of the lipoprotein: assuming that one third of ^{3}H-radioactivity at peak 11 is converted to the bound form, the ratio of ^{3}H- to ^{14}C-radioactivities of the free form in the steady state would be 4, which is in good agreement with the other proteins. Accordingly, when the outer membrane pulse-labeled for 10 seconds was treated with T4 phage lysozyme for the complete release of the bound form before SDS-gel electrophoresis, the bound form appeared for ^{14}C (steady state label) as shown by an arrow in fig. 2C and the amount of the bound form was about a half of the free form. On the other hand, a very little amount of the bound form appeared for ^{3}H (pulse-label). These results indicate that the free form of the lipoprotein which is converted to the bound form is derived from the free form pool in the outer membrane rather than that in the inner membrane or cytoplasm. Recently using immunological techniques the bound form as well as the free form of the lipoprotein was suggested to exist in the outer membrane [9].

We have previously shown [10] that the *E. coli* envelope consists of at least four major proteins (peak 4, 5, 7 and 11) which are structurally and functionally different from each other, in contrast to the single major protein shown by Schnaitman [11].

We have shown that by acidic treatment or heat treatment at 100°C peaks 4,5 and 7 form a single peak at the position closed to peak 5 [10]. Recently the similar phenomenon has been observed by the other authors [12–14]. The above results provide more evidence that the 4 major proteins in the *E. coli* envelope (in the outer membrane) are distinct: their mRNAs have different stabilities, and their assembly mechanisms are different.

Acknowledgements

We thank Dr. N. Arnheim and Dr. A Hirashima for reading the manuscript. This research was supported by grants from the U.S. National Institute of Health (GM 19043–02) and the American Cancer Society (BC-67).

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